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ORIGINAL PAPER

Oxytocin Exposure in Labor and its Relationship with Cognitive Impairment and the Genetic Architecture of Autism

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Abstract
Whether there is a relationship between oxytocin (OXT) use in labor and the risk of autism (ASD), and the nature of such relationship, is unclear. By integrating genetic and clinical data in a sample of 176 ASD participants, we tested the hypothesis that OXT is a marker for abnormal prenatal development which leads to impairments in the process of labor. OXT-exposed ASD had more obstetric complications (P = 0.031), earlier onset of symptoms (P = 0.027), poorer cognitive development (P = 0.011), higher mutation burden across neurodevelopment genes (P = 0.020; OR = 5.33) and lower transmission of polygenic risk for ASD (P = 0.0319), than non-exposed ASD. OXT seems to constitute a risk indicator rather than a risk factor for ASD, which is relevant for diagnostic and genetic counselling.

Keywords Autism · Obstetric conditions · Oxytocin exposure in labor · Genetics · Cognition

Introduction

Autism Spectrum Disorders (ASD) form a group of neurodevelopmental disorders characterized by persistent deficits in social communication and social interaction and the presence of restricted and repetitive patterns of behavior, interests, and activities (American Psychiatric Association, 2013). Although intellectual disability and language impairment are not core symptoms of ASD, they are significantly associated with the prognosis of ASD patients. Therefore, intellectual and language ability are now considered relevant clinical specifiers that need to be added to a diagnosis of ASD in the DSM.5 (Dietz et al., 2007).

Genetic and environmental factors interact in the etiopathology of ASD (Parellada, Boada, et al., 2013; Parellada, Penzol, et al., 2013). Common prevalence figures range between 8 and 17 cases per 1000 in the general population (Baxter et al., 2015), (Baio et al., 2018), (Fombonne, 2018). Much of the variance in the rapid increase in prevalence of ASD in recent decades has been attributed to increased awareness among professionals and the general population, along with broadening of the concept of autism and diagnostic accretion (Fombonne, 2018). Additionally, questions have arisen concerning the effect of modern environmental factors that may contribute to increased rates of deviant development and the presence of autism phenotypes (Emberti Gialloreti et al., 2019). Compelling evidence supports the association of obstetric variables with risk of ASD. Some of these obstetric risk factors have been identified as low birth weight, increased duration of gestation, induced labor, and intrapartum hypoxia (Hadjkacem et al., 2016; Kolevzon et al., 2007; Modabbernia et al., 2017; Smallwood et al., 2016; Wang et al., 2017). Also, oxytocin exposure in labor has also been associated with an increased risk of ASD

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and cognitive impairment (Lønfeldt et al., 2019), although the direction of the association has not been robustly determined. These obstetric complications seem to act as risk factors not only for autism and other neurodevelopmental disorders per se, but also for the severity of the condition within the autism phenotype (Wallace et al., 2008). The literature supporting this association is still contradictory, and genetic or environmental vulnerability remains a confounding issue in those observational studies (Hadjkacem et al., 2016; Lønfeldt, Nicole N. et al., 2019).

With regards to genetic factors, ASD are highly heritable, with twin and family studies suggesting heritability estimates of around 60–90% (Colvert et al., 2015; Sandin et al., 2017). Both common (Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017; Gaugler et al., 2014; Grove et al., 2019) and rare variations (De Rubeis et al., 2014; Sanders et al., 2015; Satterstrom et al., 2020) contribute to risk for ASD and may act additively (Weiner et al., 2017). However, while de novo protein disrupting mutations have been associated with more severe clinical impairment, including intellectual disability (Iossifov et al., 2014) and neurological comorbidities (Buja et al., 2018; Xiong et al., 2019), common predisposing variation is more prevalent in high functioning ASD patients; in addition, these factors do predict better cognitive performance and educational attainment (EA) in the general population (Bulik-Sullivan et al., 2015; Clarke et al., 2016).

In this study, we test the hypothesis that the use of exogenous oxytocin may act as an early indicator of abnormal prenatal development that leads to an impaired labor process. To this end, we aimed to study the association between oxytocin exposure during labor, genetic susceptibility, and general developmental delay in ASD subjects. Specifically, 176 ASD trios with collected maternal obstetric information and available exome sequencing were analyzed. We evaluated whether oxytocin exposure was associated with cognitive performance and clinical severity in an ASD cohort. Then, in a subset with available genetic, obstetric, and developmental data, rare de novo damaging mutations and polygenic scores (PGS) were analyzed to compare the genetic architecture of ASD cases with and without exposure to oxytocin during labor (N = 143).

Methods

Participants

ASD subjects were recruited at AMITEA (Comprehensive Medical Care-Autism Spectrum Disorders) (Parellada, Boada, et al., 2013; Parellada, Penzol, et al., 2013) in the Department of Child and Adolescent Department of Hospital General Universitario Gregorio Marañón, Madrid, Spain. The inclusion criteria for this study were as follows: i) a clinical diagnosis of ASD, ii) greater than or equal to 3 years of age, iii) Spanish as mother tongue, and iv) availability of data regarding oxytocin use during labor through a site-designed structured interview with the mother. In order to help mothers memorize how their labor was conducted, the questionnaire included detailed information of what labor induction with oxytocin consisted of. The study was approved by the hospital's Clinical Research Ethics Committee, and written informed consent was obtained from each participant and/or their legal guardians, as appropriate. All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

The diagnosis of ASD was assessed according to the clinical judgment of consultant child and adolescent psychiatrists, following the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision, and Fifth Edition (DSM-IV-TR and DSM.5) (American Psychiatric Association, 2013) after a full developmental, medical, and psychiatric history, review of previous educational, medical, and psychological reports, and non-structured observation. The Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2012) and/or the Autism Diagnostic Interview, Revised (ADI-R) (Rutter et al., 2003) were administered by different clinicians with training in these assessment tools (Volkmar et al., 2014). In cases where both assessments resulted in different initial conclusions, consensus meetings were organized to clarify the differences and reach a more reliable diagnosis. An in-site devised systematic interview was then conducted by a research psychologist. This interview included: demographic data (age, sex, and ethnicity) and early clinical presentation, including age at onset of ASD symptoms and presence/absence of autistic regression; obstetric data and complications, collected with the Murray-Lewis Obstetric Complications Scale (Lewis et al., 1989); oxytocin use during labor, after explaining to mothers exactly what this is or when this drug is administered, to avoid confusion with epidurals.

A previous measurement of cognitive competence was accepted if obtained by standard scales with a mean of 100 and a standard deviation of 15. These included intelligence quotient (IQ) using the Wechsler scales (Merchán-Naranjo et al., 2012; Wechsler, 2003, 2008), Leiter International Performance Scale (Leiter-R) (Leiter, 1948), or Kaufman Brief Intelligence Test (K-BIT) (Kaufman & Kaufman, 1990) (N = 99), and if IQ testing was not feasible due to absence of language skills or young age, a cognitive development index using the Merrill-Palmer (Roid & Sampers, 2004) or Brunet-Lezine scales (Josse, 1997) (N = 15).

Severity of autism symptoms was taken from calibrated ADOS severity scores (CSS) derived from the ADOS scores in those who had had the scale administrated (N = 113).

For statistical purposes, we converted all predictor variables into 8 dichotomous variables: parental and obstetric characteristics (obstetric complications and in vitro fertilization), early clinical presentation (current language level, age at first symptoms, developmental regression, and epilepsy), and autism severity outcomes (calibrated ADOS severity score and intellectual disability). We also created accumulated severity variables grouping all parental, obstetric, clinical, and autistic variables to evaluate their cumulative influence ("severity load"), and divided the group of participants in those with "0", "1–2" or "3—more of them".

We included 176 ASD patients with available obstetric data and early developmental information. Exome sequencing was available for 143 of the participants and their parents, from which blood samples for these genetic analyses were collected at the moment of recruitment. Of these, 78 participants had been exposed to oxytocin-induced labor (OXT) and 65 had not (non-OXT). Information on cognitive competence and severity of autism symptoms from standard tests was available for 114 ASD participants.

Exome Sequencing Data

The samples of Spanish ASD trios used here in this study were sequenced as part of the Autism Sequencing Consortium (ASC). Exome sequencing was performed at the Broad Institute Genomics Platform using an *Illumina HiSeq* sequencer. Variant calling was performed at the entire consortium dataset as explained by its main publication (Satterstrom et al., 2020). Briefly, raw sequencing data were processed using the Genome Analysis Toolkit (GATK) (McKenna et al., 2010) to produce variant files in VCF 4.1 format. Variant call accuracy was estimated using the GATK Variant Quality Score Recalibration (VQSR) approach. Calls with a depth of coverage less than 10 or greater than 1,000 were filtered out.

Polygenic Score (PGS) Calculation

Polygenic risk scores (PGS) were calculated for the 143 Spanish ASD trios with available exome data. Firstly, exome based VCF files were imputed on the Michigan Imputation Server (https://imputationserver.sph.umich.edu/) using 1000 Genomes Project phase 3 v.5 reference panel in order to capture genomic variants outside the exome.

PGS scores were then calculated using the imputed data. Briefly, GWAS *summary statistics* from a previous ASD study (Grove et al., 2019) were used as a discovery sample (Supplementary Table 1). An ASD PGS was assigned to each individual from the target sample consisting of the sum of the number of effect alleles carried by that sample, weighted by its effect in the discovery sample as the logarithm of the odds ratio. Only biallelic variants with an imputation quality score > 0.9 and a minor allele frequency (MAF) > 0.1% were included. Indels and single-nucleotide polymorphisms (SNPs) at ambiguous genomic positions (A/T and C/G genotypes) were also excluded. Due to the extremely complex linkage disequilibrium (LD) pattern, genetic variants within the major histocompatibility complex (MHC) were removed (from 26 to 33 Mb of chromosome 6). Correlated variants due to linkage disequilibrium were pruned using the PLINK v1.9 clumping algorithm, using an r2 = 0.1 and a window size of 500 kb.

For each subject, PGS were generated using six different P-value thresholds from the discovery sample ($P < 0.01$, 0.05, 0.1, 0.2, 0.5, and 1) in the discovery dataset for inclusion of SNPs in the PGS and calculated polygenic transmission disequilibrium test (pTDT), using PGS information from parent–child trios, as previously performed in ASD (Gonzalez-Peñas et al., 2020; Weiner et al., 2017). Briefly, the expected PGS distribution of the offspring is compared with the average PGS distribution of the parents and its deviation is tested with a one-sample t-test. Negative and positive pTDT values reveal less or more inherited polygenic variation than what is expected by chance. Under a neutral scenario, pTDT distribution may not be greater or less than 0. For each disorder, the P threshold with the highest estimated value of transmission from pTDT in the whole sample was selected and thereafter used for comparison between OXT and non-OXT ASD trios (Supplementary Table 1). Given the reported psychiatric comorbidities between ASD and other psychiatric conditions (Gonzalez-Peñas et al., 2020; Grove et al., 2019; Martin et al., 2014; Weiner et al., 2017) and the strong genetic correlations between ASD and other neuropsychiatric disorders such as major depression (MDD; $r_G = 0.412$; $P < 10^{-24}$), schizophrenia (SCZ; $r_G = 0.212$; $P < 10^{-24}$), attention-deficit/ hyperactivity disorder (ADHD; $r_G = 0.360$; $P < 10^{-11}$) (Grove et al., 2019) (Gonzalez-Peñas et al., 2020), and other phenotypes such as educational attainment (EA), we explored polygenic transmission of these four related phenotypes: ADHD, SCZ, MDD, and EA. The pTDT analysis was repeated using summary statistics from these ASD-related phenotypes (Supplementary Table 1): ADHD (Martin et al., 2018), SCZ (Ripke et al., 2014), MDD (Wray et al., 2014), and EA (Okbay et al., 2016).

Rare de Novo *Variant Analysis*

Rare variation across the 143 trios was extracted from sequencing data generated by the Autism Sequencing Consortium (ASC). For our rare variant analysis, we selected protein truncating de novo rare variation (dnPTV): nonsense,

frameshift, or splicing variants in loss-of-function (LoF) intolerant genes (probability of being a loss-of-function intolerant gene (pLI) > 0.9) and present in ExAC [http://exac.broadinstitute.org/] or the 1000 Genomes (1 KG) database [http://www.internationalgenome.org/] at an allelic frequency less than 0.1% in the European population. We also considered dnPTV those de novo missense variants in LoF intolerant genes, not present in ExAC or 1 KG and with a missense pathogenic score of MPC > 2, as this value of missense pathogenicity has been equated to that of LoF variants (Kosmicki et al., 2017; Samocha et al., 2017). Furthermore, the secondary analysis was restricted to the 208 candidate genes with important implications during neurodevelopment (NDD genes) containing de novo exonic variation (Stessman et al., 2017) or the 102 ASD predisposing genes from the recent ASC publication (Satterstrom et al., 2020). To assess the impact of rare disruptive variation at birth, we compared presence/absence of dnPTV across subjects in OXT and non-OXT trios.

Functional profiling of intolerant genes (pLI > 0.9 / MPC > 2 at MAF = 0.1%) harboring dnPTV in OXT and non-OXT trios was performed by Gene Set Enrichment Analysis (GSEA) across Gene Ontology biological processes using the ConsensusPathDB database (http://cpdb.molgen.mpg.de/; (Kamburov et al., 2013)). To avoid bias due to low-coverage genes, all genes containing rare genetic variation (N = 3085) were used as a background list for GSEA. A P-value cutoff of 0.01 and q-value (false discovery rate (FDR)-corrected P-value) cutoff of 0.05 were used. Only gene sets with more than 2 overlapping genes were included.

Protein–protein interactions (PPI) in genes harboring dnPTV in OXT and non-OXT trios were assessed using the STRING v.11.0 database (https://string-db.org/). All active interaction sources (by default in STRING) were included (Experimental, Databases, Co-expression, Co-occurrence, Neighborhood, Gene Fusion, or Text-mining). Only high-confidence interactions (score > 0.7) were included. Expected and observed connectivity values were compared in each case. Enrichment in PPIs was done for OXT, non-OXT, and ALL mutated genes separately.

Gene Expression Patterns of Disrupted Genes and Axytocin Administration

Gene expression patterns of the genes harboring dnPTV in OXT and non-OXT trios were analyzed by assessing the enrichment across brain related cell-types and their patterns of temporal expression across development. Both analyses were performed based on previously generated data of gene expression profiles throughout different brain related cell types (Zhang et al., 2016) and across brain development from Brainspan (https://www.brainspan.org/), a public database with available spatiotemporal transcriptional data in human brain development.

Enrichment of genes mutated in OXT and non-OXT trios across sets of genes specifically expressed in different brain cell types from RNAseq data (Zhang et al., 2016) was assessed using the *pSI package*. Expression values from several cell-type replicates were log2 normalized and averaged. Specificity for the five cell types was calculated with the *specificity.index* function of the *pSI* R *package*. Significance was assessed using Fisher's exact test with a specificity (pSI), followed by FDR-correction of P-values. Two different specificity indexes were included (pSI < 0.05 and pSI < 0.01) for each cell type.

Evolution of OXT and non-OXT gene expression values was studied using the available gene expression data for brain regions in the BrainSpan atlas database (https://www.brainspan.org/). The gene expression percentage (measured as reads per kilobase per million reads mapped (RPKM)) was calculated at 30 different time points across development relative to overall expression. Average prenatal and postnatal gene expression levels were also compared across genes mutated in either OXT or non-OXT participants, and pairs of observations were compared against the null hypothesis of zero variation between pre- and postnatal averaged gene expression values.

Statistical Analysis

PASW Statistics 18.0 (SPSS Inc, 2009) and R v.3.6.0 (Team, 2015) were used for statistical analyses, with a P-value significance threshold set at < 0.05.

Depending on whether the variables were continuous or discrete, descriptive or frequency analyses were performed, the results of which are provided in Table 1. Normality of clinical and cognitive variables was confirmed with the Kolmogorov–Smirnov test. Measurement variables were compared using a two-sample t-test if they were normally distributed or otherwise a Mann–Whitney test. Chi-square tests were performed to compare proportions of subjects when analyzing dichotomous severity variables. Difference in the burden of dichotomous severity variables per subject was assessed with a Cochran-Armitage trend test. Briefly, subjects were divided into those having 0, 1–2, or 3 of the severity variables, and proportions of OXT and non-OXT subjects were compared through a linear trend. The percentages of OXT and non-OXT subjects having each of the 8 dichotomous predictors were compared using a Wilcoxon signed-rank test. This way, the average deviance from 0 was tested across the 8 measured variables.

A one-sample t-test was used to assess the polygenic transmission disequilibrium of ASD, ADHD, MDD, and EA risk alleles in OXT and non-OXT trios separately, using pTDT scores for both distributions, as performed in previous

studies (Weiner et al., 2017). To overcome statistical power limitations and strengthen our findings, significant results were confirmed by conducting 1000 random permutations of OXT/non-OXT status and subsequently comparing the real pTDT deviations to the null distribution of the permuted datasets. Two sample t-test was used to compare polygenic transmission in OXT vs non-OXT trios. A two-tailed Fisher's exact test was performed to compare dnPTV burden across OXT against non-OXT participants. A Wilcoxon signed-rank test was used to compare gene expression trends between prenatal and postnatal stages across genes mutated in either OXT or non-OXT trios, separately. Two-way ANOVA was performed to assess the difference in gene expression trends in OXT vs non-OXT trios. Interaction between gene expression(prenatal/postnatal) and subject with mutated gene (OXT/non-OXT) was evaluated.

Results

Oxytocin Exposure During Labor and Developmental Delay

The sample included 176 patients with obstetric and developmental information, aged between 3 and 46 years of age (Median 11.5; Mode 7). More than 50% of them experienced one or more obstetric complications, and in nearly 56% of the participants, oxytocin was used during labor. Table 1 shows the demographic, obstetric, clinical, and cognitive characteristics of ASD individuals (N = 176) in the OXT and non-OXT trios. No differences in sex, parental age at conception, mothers' psychiatric history of anxiety and/or depression, or ethnicity were observed between OXT and non-OXT ASD groups.

Oxytocin use during labor was associated with a higher number of obstetric complications and with an onset of ASD symptoms during the first year of life. When severity variables were measured dichotomously, a significant severity bias towards OXT subjects was observed when comparing the percentage of OXT and non-OXT subjects having each severity event (Paired t-test P = 0.041; Fig. 1A). Indeed, oxytocin use was significantly associated with a higher load of severity events per subject (Cochran–Armitage trend test P = 0.013; Fig. 1B). Poorer cognitive development (IQ) was also observed in OXT subjects (Mann–Whitney-test P = 0.019; Fig. 1C).

Association Between Polygenic Risk Transmission and Axytocin Exposure

The increased presence of at least one obstetric complication ($X^2 = 4.028$ P = 0.045), an early onset of ASD symptoms (i.e., before age 1) ($X^2 = 4.225$ P = 0.040), and lower IQ (t = 0.644 P = 0.043) in the OXT group was confirmed in the subset of patients with available genetic information (N = 143). Polygenic risk (PGS) for ASD was differentially transmitted to ASD subjects in OXT and non-OXT trios (two-sample t-test; t = 2.307; P = 0.0186). Specifically, ASD PGS was significantly over-transmitted to ASD subjects in non-OXT trios (pTDT one-sample t-test; t = 2.193; P = 0.03195; Fig. 2A), but not to ASD subjects in OXT trios (one-sample t-test; t = -1.087; P = 0.280; Fig. 2A).

ADHD PGS was also differentially transmitted to ASD subjects in OXT and non-OXT trios (two-sample t-test; t = 2.663; P = 0.0086). ADHD PGS was over-transmitted in Non-OXT trios (one-sample t-test; t = 2.667; p = 0.0097; Fig. 2B) but not in OXT trios (one-sample t-test; t = -0.89; P = 0.375; Fig. 2C), in a pattern similar to ASD PGS. No significant over-transmission was found for the remaining assessed phenotypes (SCZ, MDD, and EA). No significant correlation was observed between pTDT values from assessed phenotypes (ASD, ADHD, SCZ, MDD, and EA) (Fig. 2C). All significant results were confirmed by permutation tests (Supplementary Table 2; permutation-P_{ASD} = 0.0159; permutation-P_{ADHD} = 0.0099).

Association Between Rare Disruptive Genetic Variation and Axytocin Administration

We identified 44 mutation-intolerant genes (pLI > 0.9 / MPC > 2) with 62 de novo protein truncating variants (dnPTVs) in ASD individuals with available exome sequencing data (N = 143) (Fig. 3A). Of those, 33 genes, presenting 40 dnPTVs, were mutated across ASD individuals in OXT trios (N = 29; 0.51 dnPTV per subject) and 17 genes, 22 dnPTVs, across ASD individuals in non-OXT trios (N = 19; 0.34 dnPTV per subject).

When all dnPTVs were included, no difference in dnPTV burden between OXT and non-OXT trios was described (Fisher's exact test P = 0.375; OR = 1.43; Table 2). However, a significant number of PPI were described in genes mutated in OXT trios ($P = 1.94 \times 10^{-3}$; Supplementary Table 3), a pattern not observed in non-OXT trios (P = 0.244). Although genes harboring dnPTV in both OXT and non-OXT trios were found to be mainly enriched across chromatin remodeling pathways, only genes mutated in OXT trios were described to be enriched in learning or memory ($P = 4.85 \times 10^{-4}$) and cognition pathways ($P = 1.23 \times 10^{-3}$; Supplementary Table 4).

The mutation burden was then restricted to genes harboring protein truncating variation in ASD (Satterstrom et al., 2020) and, independently, to genes with profound involvement in neurodevelopment (NDD) (Stessman et al., 2017). We found a significant enrichment in NDD genes in ASD individuals exposed to oxytocin (11 of 78 OXT ASD individuals) relative to those not exposed (2 of 65 non-OXT

Table 1 Description of demographic, obstetric, and autism severity characteristics of the analyzed ASD cohort (N = 176, except for severity outcomes including intellectual disability, which were available for n = 114)

% or Mean (SD)				
All subjects	OXT (N = 98)	Non_OXT (N = 78)	P-val OXT vs Non_OXT	
Demographic data				
Age	14.53 (9.89)	13.62 (8.19)	15.68 (11.63)	0.19
Progeny sex (male)	68.75	67.35	70.51	0.65
Ethnicity (European ancestry)	92.04	93.56	90.81	0.49
Parental and obstetric characteristics				
Obstetric complications, yes	53.98	61.22	44.87	**0.031**
In vitro fertilization	3.66	4.44	2.70	0.691
Early clinical presentation				
Current language level (simple phrases vs fluent)	57.95	57.14	58.97	0.81
First symptoms before age 1, yes	26.14	32.65	17.95	**0.027**
Developmental regression, yes	34.65	34.69	34.61	0.99
Epilepsy, yes	12.50	13.27	11.54	0.731
Autism severity outcomes (N = 114)				
Calibrated ADOS Severity Score (8–10)	85.71	59.02	46.10	0.18
Intelligence Quotient (IQ)	78.29 (23.59)	91.15 (24.87)	**0.019**	
Intellectual disability (IQ < 70)	32.82	40.00	25.76	0.08
Accumulated severity variables (parental, obstetric, clinical and autistic variables)				
ASD subjects with no severity variable (9/176)	-	33.33	66.67	**0.013**
ASD subjects with 1–2 severity variables (64/176)	–	46.88	53.12	
ASD subjects with 3 or more severity variables (103/176)	–	63.11	36.89	
Severity bias towards oxytocin use during labor	–	–	–	**0.041**

In each case, P-values are derived from t-tests or Mann–Whitney tests when comparing measurement variables and Chi-square tests when comparing proportions. ASD individuals were divided according to oxytocin exposure during labor into OXT (N = 98) and non-OXT (N = 78) groups. Severity bias towards oxytocin use was assessed by analyzing the percentages of OXT and non-OXT subjects having each of the 8 dichotomous predictors using a Wilcoxon signed-rank test. Difference in the burden of dichotomous severity variables per subject was assessed by the Cochran-Armitage trend test

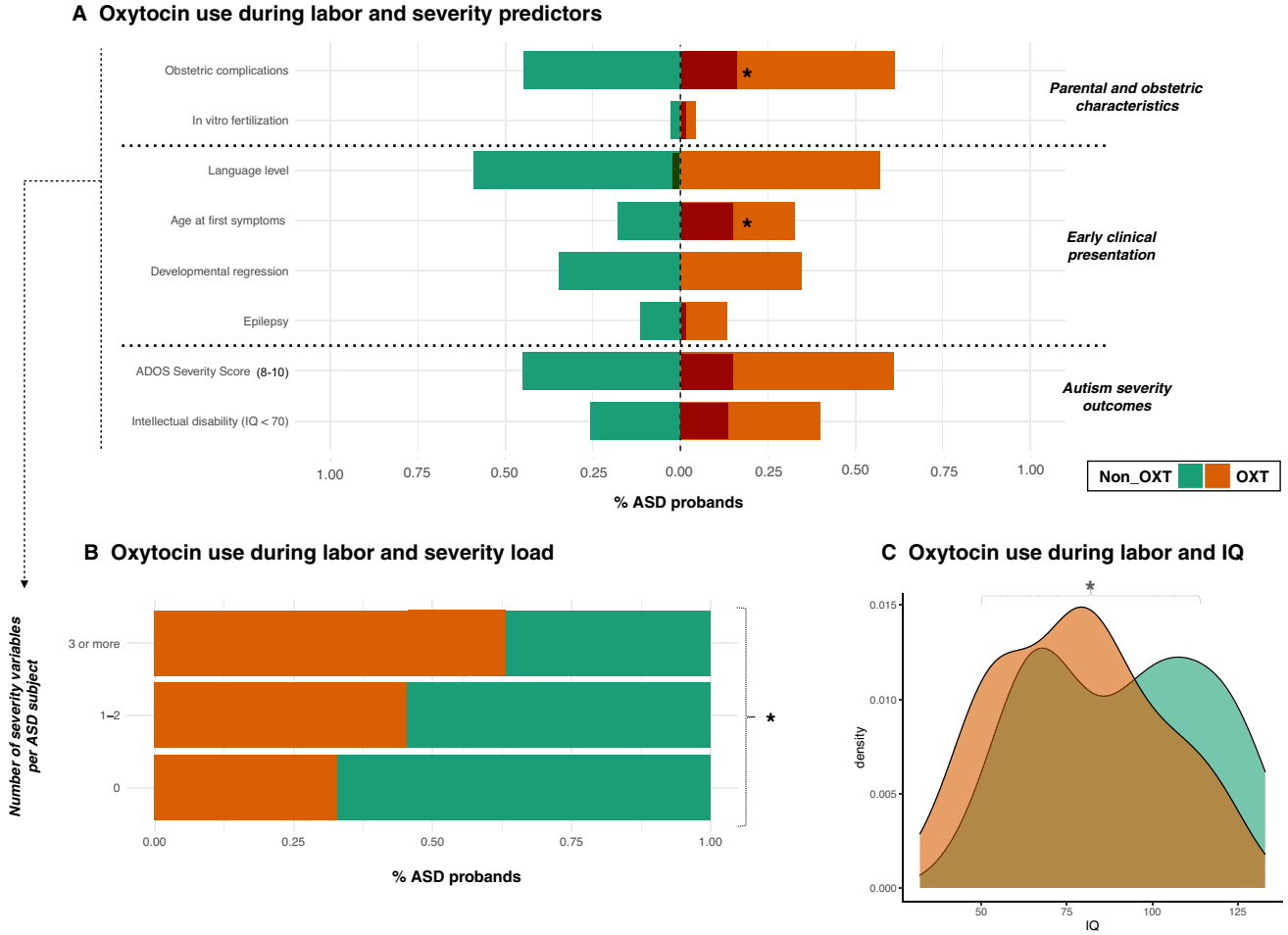

Fig. 1 Oxytocin exposure during labor and severity of outcomes. **A** The distribution of each of the 9 dichotomous severity variables was compared between ASD individuals with (OXT, in green) and without (non-OXT, in orange) oxytocin exposure during labor. Biases toward a higher percentage of ASD subjects in OXT and non-OXT trios were represented by dark orange and dark green colors, respectively. **B)** The total burden of the 9 dichotomous severity variables was compared between OXT and non-OXT trios. The percentage of participants with 0, 1–2, or 3 or more severity variables is represented in the graph. A Cochran-Armitage trend test was performed. **C)** IQ values were compared between ASD individuals in the OXT and non-OXT trios using a Mann–Whitney test. Significant values ($P < 0.05$) are indicated by "*." Abbreviations: IQ = Intelligence quotient, OXT = ASD subjects with oxytocin exposure during labor, non-OXT = ASD subjects without oxytocin exposure during labor

ASD individuals) (Table 2). Conversely, we found no significant enrichment in ASD genes.

Gene expression Patterns of Disrupted Genes andOxytocin Administration

Following the finding of a higher NDD dnPTV load in ASD subjects exposed to oxytocin, we further explored whether this observation is consistent with gene expression profiles of the mutated genes.

Whole gene expression profiles across brain development were downloaded from BrainSpan, and normalized (see Methods). Gene expression profiles for genes mutated in either OXT or non-OXT participants were represented separately (Fig. 3C). Significant difference in expression trends from prenatal to postnatal stages between genes mutated in OXT or non-OXT participants was found (two-way ANOVA; $F = 8.43$; $P = 0.0048$). Specifically, a decreasing gene expression trend, from prenatal (13 stages) to postnatal (18 stages), was observed across genes mutated in OXT participants, (Wilcoxon signed-rank test P-value $= 1.36 \times 10^{-3}$; Fig. 3D), but not across genes mutated in non-OXT participants (Wilcoxon signed-rank test P-value = 0.778; Fig. 3E).

We also assessed enrichment of genes mutated across specific brain cell types, whose gene expression patterns were previously described (Zhang et al., 2016). Interestingly, genes mutated across individuals in non-OXT trios

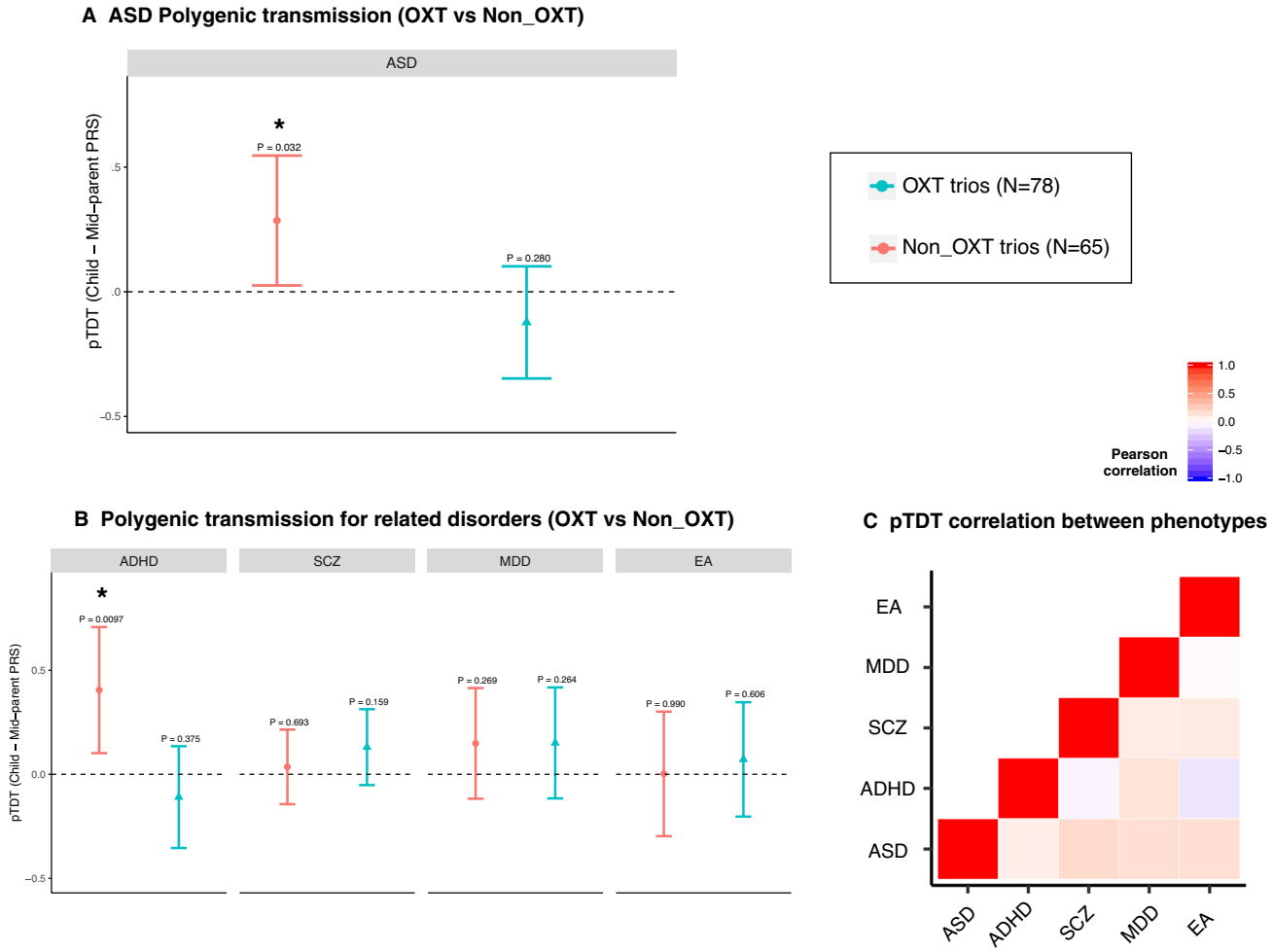

Fig. 2 Polygenic transmission in ASD trios with oxytocin exposure during labor. Disequilibrium in polygenic transmission (pTDT) of common predisposing variation from ASD and related phenotypes (ADHD, SCZ, MDD, and EA) was assessed in OXT (N = 78) and non-OXT trios (N = 65). A) Correlation analyses between pTDT values for all traits tested showing no significant correlation in the study sample. B) Results of pTDT test using ASD polygenic scores. C) Results from pTDT test using polygenic scores from related traits. Transmission disequilibrium is represented as standard deviations of the mid-parent distribution. Colored geometric lines represent 95% confidence intervals. P-values measure the probability that the mean of the pTDT deviation distribution is 0 (two-sided, one-sample t-test). Abbreviations: ASD = autism spectrum disorder, IQ = Intelligence quotient, ADHD = attention-deficit/ hyperactivity disorder, SCZ = schizophrenia, MDD = major depressive disorder, EA = educational attainment, OXT = ASD subjects with oxytocin exposure during labor, non-OXT = ASD subjects without oxytocin exposure during labor

were found to be significantly enriched in neuron-specific genes ($P_{FDR} = 0.017$ at pSI < 0.05, Fig. 3B; supplementary table 5), whereas genes mutated across OXT subjects had a less specific pattern of gene expression (Fig. 3B).

Discussion

In this study, we aimed to explore genetic and developmental differences between ASD individuals exposed to oxytocin during labor and those not exposed. ASD individuals exposed to oxytocin in labor were characterized not only by lower cognitive competence and early onset of symptoms, but also by a higher likelihood of having de novo disrupting variation in genes strongly involved in early neurodevelopment. Conversely, ASD participants not exposed to oxytocin were characterized by better cognitive abilities, higher polygenic transmission of ASD and ADHD alleles, and higher likelihood of having de novo variation affecting genes with neuronal-biased expression throughout development.

The literature describing outcomes in children exposed to oxytocin during labor has reported associations between oxytocin use, dose, and duration in labor and increased risk of an ASD outcome in the progeny (Gialloreti et al., 2014; Gregory et al., 2013; Soltys et al., 2020; Weisman et al.,

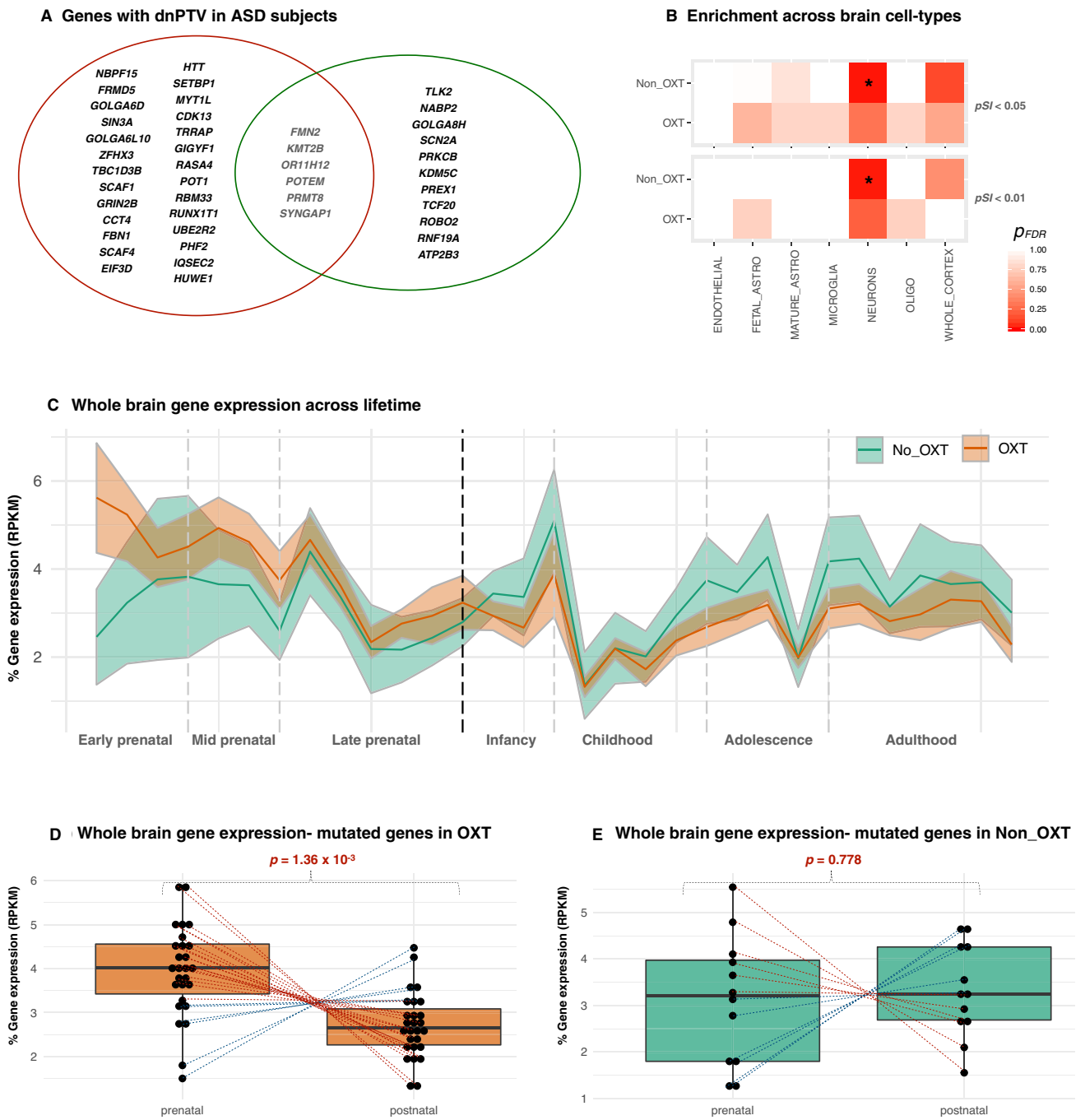

Fig. 3 Gene expression patterns of mutated genes in ASD individuals with (OXT in green) and without (non-OXT in orange) exposure to oxytocin during labor. **A** Lists of genes with dnPTV in OXT ($N_{subjects} = 78$; $N_{genes} = 33$) and non-OXT trios ($N_{subjects} = 65$; $N_{genes} = 19$). **B** Gene set enrichment across cell types in the adult human cerebral cortex (Zhang et al., 2016). Enrichment analyses were done for genes mutated in OXT and non-OXT trios using the pSI package, using two different specificity indexes (pSI < 0.05 and pSI < 0.01) for each cell type. Probability values were FDR-corrected in each case; "*" indicates significant values ($p_{FDR} < 0.05$). **C** Average expression of genes mutated in OXT and non-OXT trios during neurodevelopment. The percentage of gene expression (measured as reads per kilobase per million reads mapped (RPKM)) at each of the 30 different time points, is represented relative to the overall expression. Gene expression values were averaged across all available brain tissues at BrainSpan (https://www.brainspan.org/) at each time point. Dark lines and shaded areas represent average expression and 95% confidence intervals. The black dashed line separates prenatal from postnatal expression. The grey dashed lines split neurodevelopment into six developmental stages. **D-E** Differences between prenatal and postnatal gene expression levels for genes mutated in OXT (D) and non-OXT trios (E). Red and blue dashed lines connect prenatal and postnatal stages for genes with decreasing and increasing average expression values, respectively. Prenatal-postnatal observations were analyzed to contrast the null hypothesis whereby the difference between pairs of observations is zero with the Wilcoxon signed-rank test

Table 2 dnPTVs across ASD individuals in OXT and non-OXT trios

Number of ASD individuals with dnPTV (No. mutations/individual)					
	All sample	OXT (N = 78)	Non_OXT (N = 65)	OR	P-val
All de novo PTV	48 (0.43)	29 (0.51)	19 (0.34)	1.43	0.375
102 ASD genes (Satterstrom et al., 2020)	11 (0.08)	7 (0.09)	4 (0.06)	1.50	0.754
208 NDD genes (Stessman et al., 2017)	**13 (0.10)**	**11 (0.15)**	**2 (0.03)**	**5,33**	**0.037**

Numbers and ratio of ASD individuals with dnPTVs considering i) all de novo PTVs (row 1), ii) dnPTVs in genes harboring truncating variation in ASD (row 2), and iii) dnPTVs in genes harboring truncating variation in neurodevelopmental disorders (NDD) (row 3) in OXT and non-OXT trios are shown. Two-tailed Fisher's exact tests were performed for each set of genes. Only mutation-intolerant genes were included in the analyses (pLI > 0.9 / MPC > 2). Abbreviations: dnPTV = de novo protein truncating variant, ASD = autism spectrum disorder, NDD genes = genes with important involvement during neurodevelopment (Stessman et al., 2017), pLI = probability of being a loss-of-function intolerant gene, MPC = missense pathogenic score, OXT = ASD subjects exposed to oxytocin use during labor, non-OXT = ASD subjects not exposed to oxytocin use during labor

2015), at least in males (Soltys et al., 2020; Weisman et al., 2015). However, the results are not consistent (Guastella et al., 2018; Soltys et al., 2020), and the effect of the association seems weak (HR = 1.05) after adjusting for many confounding factors (Lønfeldt, Nicole N. et al., 2019; Lønfeldt et al., 2020; Stokholm et al., 2020). For instance, the association was shown to disappear in a large Danish register-based cohort study, in which the analyses were adjusted for history of psychopathology, antidepressants during pregnancy, and socioeconomic factors, among others (Lønfeldt, Nicole Nadine et al., 2020). Moreover, Oberg et al (2016) in a cohort of more than 1 million births in Sweden showed that comparing siblings whose births were discordant for induction, labor induction was no longer significantly associated with ASD risk. Many different study designs, mechanistic studies and an increased cross-talk between obstetrics and neuroscience is needed to make significant progress in the understanding of the relationships between obstetric factors and risk of ASD (Kenkel et al., 2019).

The epidemiological studies so far have typically used a descriptive approach, with associations reported but no hint with respect to the direction of the relationship or the mechanism involved in that relationship (Modabbernia et al., 2017), indicating the need for further investigation to try to clarify the direction and mechanisms of the association.

On the one hand (Gottlieb, 2019a; Oberg et al., 2016; Simpson & Atterbury, 2003), it has been speculated that the use of synthetic oxytocin during labor may result in desensitization and downregulation of oxytocin receptors in the fetal brain (Gottlieb, 2019a, 2019b; Oberg et al., 2016), leading to abnormal development and subsequent increased rates of ASD in the affected children. However, this hypothesis is countered by the fact that many studies have reported similar associations between autism and cesarean section (Yip et al., 2017) or epidural analgesia (Qiu et al., 2020). With the data herein presented we cannot make any statement regarding whether oxytocin use increases or not the risk of ASD. Nevertheless, we show that when studying such association it is necessary to consider the presence of obstetric complications and early markers of deviant development. Indeed, in this study, we report an association between exposure to oxytocin during labor and other obstetric complications in our sample. On the other hand, a genetic factor predisposing to both obstetric complications and altered neurodevelopment could be behind the reported associations. For example, a rare-genetic ASD cohort with disruptive mutations in *CHD8* (an ASD candidate gene proposed to strongly affect early neurodevelopment) showed that up to 40% had been born after induced labor and, in addition, 33% had had a cesarean birth (Bernier et al., 2014); both data doubling the expected percentages (Simpson & Atterbury, 2003). Needless to say, caution should be exercised in the use of oxytocin for labor induction, as it has been consistently shown that there is an association between the use of oxytocin and uterine hyperstimulation, which may have adverse consequences on the outcome of labor and subsequently on development (Clark et al., 2009; NICE guidelines, 2014).

In this study, we explored the possibility that children with very early (intrauterine) developmental difficulties are involved in at-risk labors during which oxytocin is more frequently prescribed to help delivery. We reported genetic architectures among ASD subjects who had been exposed to oxytocin (OXT) differentiating them from those who had not (non-OXT). Our results suggest that OXT subjects have a more severe phenotype likely associated with disruption of NDD genes and lower contribution of polygenic load whereas non-OXT subjects have a higher contribution from polygenic background. It should be noted that this more severe phenotype present in the OX sample comprises the accumulation of the effect of different variables; although when studying them separately we did not see differences in all of them, in particular ASD-specific severity, measured by ADOS, did not show differences. Therefore, we would speak of a severity more defined by a delay in the development of early childhood milestones, as well as cognitive. Recent evidence suggests that genetic architecture in ASD and NDD could be based on an oligogenic model (Iakoucheva et al., 2019) in which risk conferred by disrupting mutations in *core* genes (Liu et al., 2019) is influenced by genomic (polygenic) background. The literature has widely demonstrated the contribution of both common (Autism Spectrum Disorders Working Group of The Psychiatric Genomics Consortium, 2017; Gaugler et al., 2014)

and rare (Grove et al., 2019) genetic variation in the risk of autism (Weiner et al., 2017). However, it has been recently described that common predisposing variation to NDD and ASD are far from correlated (Niemi et al., 2018), highlighting the distinction between NDD and ASD and the challenges of disentangling the genetic heterogeneity in coexisting ASD subtypes (Gonzalez-Peñas et al., 2020; Hong et al., 2020).

Regarding common genetic variation, we have described significant polygenic contribution from ASD and ADHD in non-OXT ASD trios. ASD heritability estimates from common variation has been calculated to account for up to 50% of the phenotypic variance (Gaugler et al., 2014), and several studies have found that the PGS for ASD (Anttila et al., 2018; Clarke et al., 2016) and NDD (Niemi et al., 2018; Sniekers et al., 2017) are directly and inversely correlated with higher IQ in the population, respectively. Based on our data, a *specific* autistic phenotype, characterized by better cognitive development and higher polygenic burden, emerges after removing those subjects exposed to oxytocin. Conversely, non-significant polygenic transmission for any of the disorders assessed has been described in OXT subjects.

With regard to rare variation, we report higher prevalence of de novo protein disrupting variants in OXT than non-OXT subjects. The difference in mutation rate between OXT and non-OXT participants is significantly higher when restricting this to broad NDD genes, but not to specific ASD genes. These results are in line with those from the latest ASD exome sequencing study to date (Satterstrom et al., 2020), in which the top 102 ASD risk genes were divided into NDD and predominant ASD genes, which found that the former group of genes was associated with lower IQ and higher symptom severity.

All dnPTVs affecting OXT trios were distributed across significantly interconnected genes and with prenatal biased expression patterns, while genes mutated in non-OXT subjects were enriched in neuron-specific genes. These observations are consistent with recent results suggesting that ASD risk genes associated with neuronal communication are not more expressed during prenatal stages, as occurs with genes related to expression regulation or chromatin remodeling (Satterstrom et al., 2020). Moreover, chromatin remodeling genes have been extensively related to severe neurodevelopmental conditions apart from ASD (Barnard et al., 2015; Cotney et al., 2015; Ronan et al., 2013).

These functional patterns can be exemplified with some of the genes here described. Among the genes affected in OXT subjects, GRIN2B, encoding a member of the N-methyl-D-aspartate (NMDA) receptor family, has been found to be related with neurodevelopmental conditions (Hu, Chen, Myers, Yuan, & Traynelis, 2016; Myers et al., 2019; Platzer et al., 2017). GRIN2B is one of the main ASD risk genes with comorbid intellectual disability (ID) and severe neurodevelopmental affectation recently described in the largest ASD exome study to date (Satterstrom et al., 2020). Moreover, mutations within MYT1L, IQSEC2 or HUWE1, also affected in OXT subjects, have been extensively described as ID risk factors (Mayo et al., 2015; Moortgat et al., 2018; Shoubridge et al., 2010; Stevens et al., 2011). On the other hand, ATP2B3 and PRKCB, the genes affected in subjects from non-OXT trios with the largest bias towards postnatal gene expression, have been related with ASD (Toma et al., 2011; Wen, Alshikho, & Herbert, 2016) but their evidence behind neurodevelopmental affectation is scarce.

We note several important limitations of our study. First, we do not have trios without ASD, nor undiagnosed siblings of our subjects, nor a control group; therefore, a direct comparison of the risk of ASD associated with the use of oxytocin during labor is not possible in our study. Second, data on oxytocin use during labor was obtained by mother self-report using a custom-designed questionnaire, which may be subject to several biases including a recall bias. Also, the questionnaire could have been more comprehensive, including the use of other drugs during labor, such as prostaglandins or for epidural anesthesia. However, these were not initially included because they constituted more a standard practice than the use of oxytocin. Due to the nature of the retrospective data collection, the dose(s) of exogenous oxytocin used in the obstetric procedures was not available. Another limitation is the lack of a direct measure of obstetric complication such as a blood analysis of the umbilical cord for the measurement of oxygen supply. This type of measurement was not a standard practice at the time of the delivery of the participants included herein. Furthermore, as we have an average 9% of non-European ancestry in our ASD cohort, we are aware of the implications for polygenic score prediction based on GWAS discovery data from European cohorts (Grove et al., 2019) in terms of loss of statistical power (Duncan et al., 2019). However, pTDT has been used to deal with population ancestry since analyses are based on parental transmission of predisposition variants (De Rubeis et al., 2014). Finally, although de novo truncating variants in NDD genes have been demonstrated to be highly pathogenic (Grove et al., 2019), other structural variants such as CNVs were not assessed in this study. Therefore, our results should be interpreted with caution, and larger sample sizes from other ASD cohorts with available information on oxytocin use during labor and genetic data are needed to replicate our findings.

In conclusion, our results argue against the notion that oxytocin exposure during labor is causally related to a greater prevalence of ASD. Instead, oxytocin exposure during labor is more likely related to a subtype of ASD with a greater load of rare de novo disrupting genetic variation and developmental deviation. We encourage the research and clinical community to replicate our findings and to include information on oxytocin exposure during

labor as a potential risk marker for greater neurodevelopmental impairment and poorer cognitive competence in autism, which may guide therapeutic targeted interventions and prioritization of genetic diagnosis and counseling in this subgroup of ASD individuals.

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Author Contributions A.G.A., J.G.P., and M.P. conceived and designed the study; M.J.P., P.H., C.M., M.P., E.W., and A.GA. recruited and evaluated the participants; A.G.A and J.G.P. performed most of the data analysis with great input from C.D.C.; X.G. and X.C. significantly contributed to the genetic analyses and discussion; A.G.A. and J.G.P. wrote the first draft of the manuscript; and all authors read, reviewed, and approved the manuscript.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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